Video Article

Measuring Exocytosis in Neurons Using FM Labeling

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Abstract

The ability to measure the kinetics of vesicle release can help provide insight into some of the basics of neurotransmission. Here we used real-time imaging of vesicles labeled with FM dye to monitor the rate of presynaptic vesicle release. FM4-64 is a red fluorescent amphiphilic styryl dye that embeds into the membranes of synaptic vesicles as endocytosis is stimulated. Lipophilic interactions cause the dye to greatly increase in fluorescence, thus emitting a bright signal when associated with vesicles and a nominal one when in the extracellular fluid. After a wash step is used to help remove external dye within the plasma membrane, the remaining FM is concentrated within the vesicles and is then expelled when exocytosis is induced by another round of electrical stimulation. The rate of vesicles release is measured from the resulting decrease in fluorescence. Since FM dye can be applied external and transiently, it is a useful tool for determining rates of exocytosis in neuronal cultures, especially when comparing the rates between transfected synapses and neighboring control boutons.

Protocol

Cells: Rat (or mouse) primary hippocampal neuronal cultures (14-28 days in vitro).

Stimulation: Electrical, delivered via two platinum electrodes; 70-90 mV

Microscopy: 60x oil lens on an inverted CCD fluorescent microscope.

Software: Slidebook (Intelligent Imaging Innovations, Santa Monica, CA)

See supplemental methods page for further details

1. Warm HEPES-buffered saline (HBS) to room temperature. Add the glutamate receptor antagonists APV (50 µM final concentration) and CNQX (10 µM final). Each FM experiment usually requires 20-30 mL HBS. For working concentration of 10 µM FM 4-64 (Molecular Probes), dilute stock solution 1:1000 in HBS (with antagonists added). Make 2 mL FM soln for each experiment. Cover solution with foil to prevent light exposure.
2. Mount the coverslip containing the neurons onto the electrode chamber. Check that media in chamber is level with top of chamber and completely covers the electrodes. Remove any excess solution from bottom of coverslip. Add oil to the lens (if using an oil lens).
3. Set up the perfusion apparatus. First rinse with a few mL of each the following in the given order (let each one flow thoroughly before adding the next): water, ethanol, water, then HBS. Next add the HBS (save a few mL to add by hand in future steps) and close the perfusion. Set up the perfusion outlet and the suction on opposite sides at the edges of the coverslip. It is best if the perfusion outlet reaches into the chamber, where as the suction should rest at the very top. While adding HBS (either by perfusion or by hand), open the suction and adjust its position so that it maintains the media at the right level- just fully covering the electrodes.
4. Look for the area on the coverslip you wish to image and try to roughly bring it in focus. Optimal areas contain many synapses, but should not be so dense such that individual processes become indistinguishable. There should be no-to-minimal cell bodies, extraneous membranes (such as clumps of astrocytes), or other nonspecific materials (such as lint). The FM dye may nonspecifically adhere to these.
5. Use the "WG" filter cube (which excites with green light and collects red-to-far red emissions). Set Slideview "capture preferences" to deliver 900 AP at 10Hz upon onset of image 0 (typically 300 to 900 APs are used for this loading stimulus). In the image window, select the "fluo-vis-red" filter settings and change exposure time to 100 ms. Turn off any pre-existing timelapse settings. Do not take any images until you are ready to start stimulation.
6. Make sure the suction is on/open. Quickly add the FM soln (2 mL) to the chamber at the opposite end of the suction. Immediately press okay in the image window to take an image and begin stimulation. Wait 30-45 sec after stimulation and then quickly wash out FM by adding ~2 mL of HBS (I do this by hand with a pipette, but this can also be done by perfusion).
7. Wash the FM with HBS via perfusion for ~10 minutes. The flow rate should be 1-1.5 mL/min. During this wash step, change the stimulation preferences so that the onset of stimulation begins @ image 10 (typically 900 to 1200 APs @10 Hz are used for this destaining stimulus). About 7 min into the wash, double check the preferences (onset of stimulus at image 10). Now using the small focus window choose and focus a subregion. Make sure the stimulation settings have been changed before proceeding to next step. Take a single image to see if the wash is complete (individual synapses should be clearly punctated). If not, increase the flow rate an additional 0.5mL/min, and wait another 2 minutes.
8. Once wash is complete, adjust the exposure time as necessary to still receive a good, clear signal (usually between 50-100 ms. Exposure times should be minimized due to rapid photobleaching of the FM). Then set the timelapse preferences to take 38 images every 5 seconds. Check the perfusion to make there is enough HBS for another 5 minutes, and leave perfusion flowing. Start timelapse to begin destaining.
9. After destain, change stimulation settings to deliver 1200 AP @ 10Hz (typically 1200 to 2000 APs are used for this final step) upon image 0. Turn off the timelapse settings and take a single image to begin stimulation. The perfusion should still be flowing This step will help remove any remaining vesicular FM dye, in order to determine baseline/"total releasable" fluorescence. After the stimulus is finished, turn off the stimulation settings, bring the region into focus. Close the perfusion and suction, and then take two final baseline images. Either single images or Z-stack images (0.5 µm steps) can be taken. Z-stacks are useful in case there was a shift in the plane of focus during the experiment.

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10. Your FM experiment is done. If immediately doing more experiments, the lens and chamber should be cleaned between each trial, but the perfusion apparatus need not be cleaned until after the final experiment. Rinse the apparatus completely with water, then ethanol.

Discussion

As 300 action potentials (APs) is sufficient to induce at least one round of vesicle recycling, a loading stimulus of 300 APs or more is often given to label the recycling pool of vesicles. Though more intense loading stimuli, such as 900 APs, may allow a nominal number of additional vesicles to be labeled, this also increases the amount of time the dye can embed in extracellular membranes, leading to greater non-specific staining. I have found the wash step to be critical to ensure proper destaining. It is important to perfuse with flow rate 1 to 1.5 mL per minute, usually for 10 + 2 minutes. A single image can be taken ~7 minutes into the wash to monitor the extent of dye removal. If necessary, the flow rate and or wash time can be slightly increased. For our experiments it was undesirable for the wash to proceed greater than 10-12 minutes as this increases the likelihood of losing vesicle-labeling through spontaneous exocytosis events which may occur even when the cell is at rest. For the destaining step, 900 to 1200 APs were administered to release all the labeled vesicles. Additionally another 1200 to 2000 APs were often given to obtain a baseline value of "releasable fluorescence", used to normalize the destaining data.

References

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